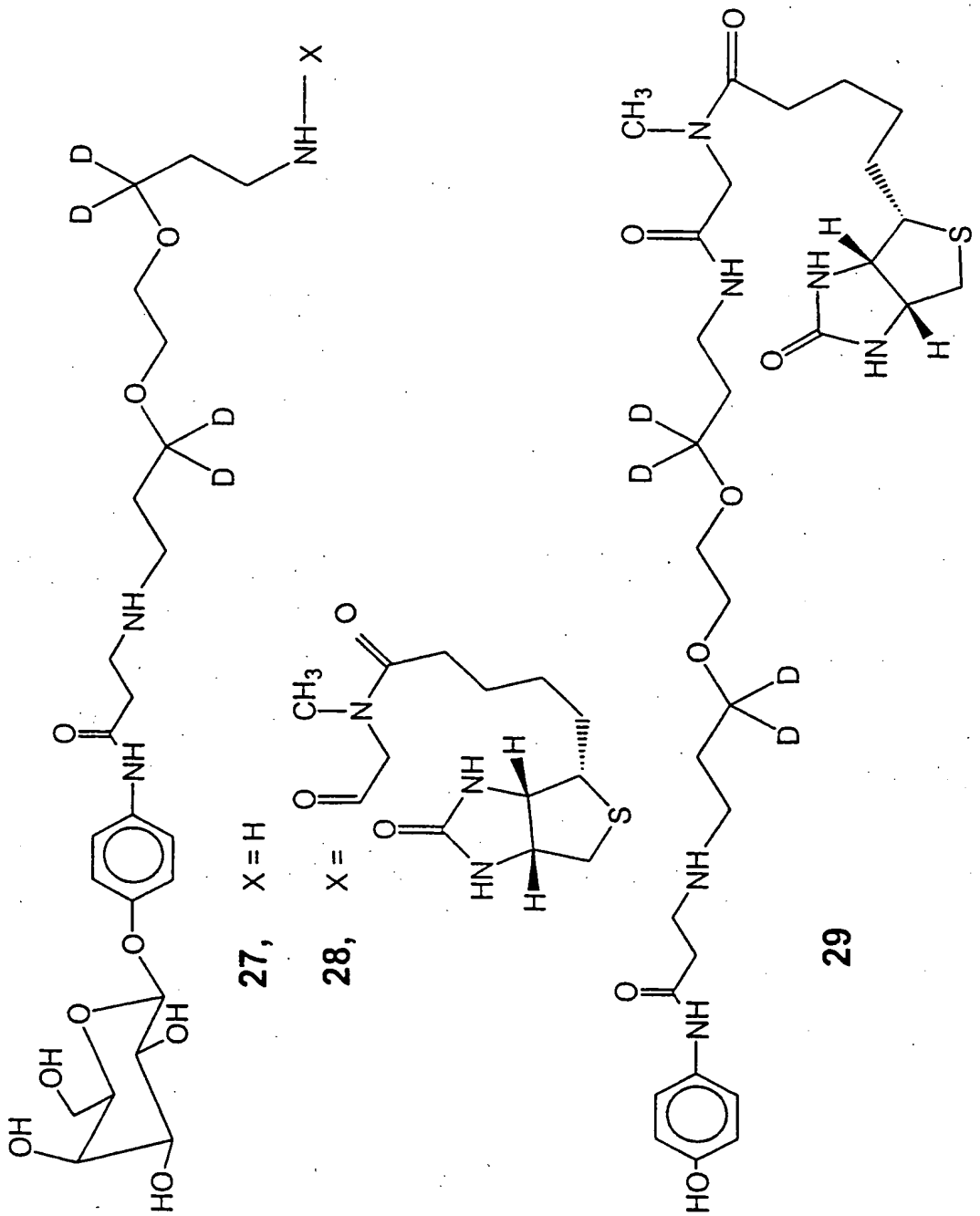
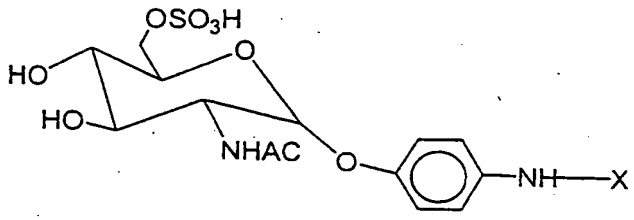
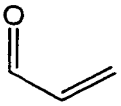
Scheme 19



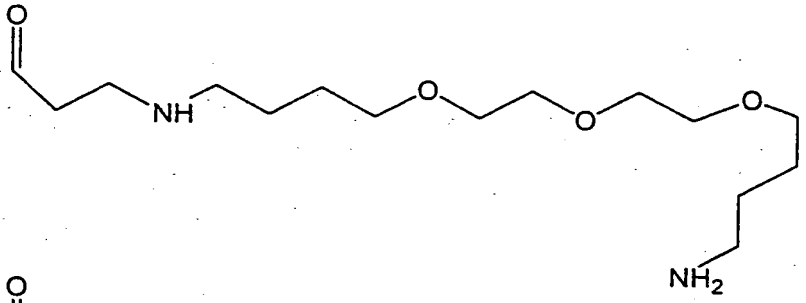
Scheme 20



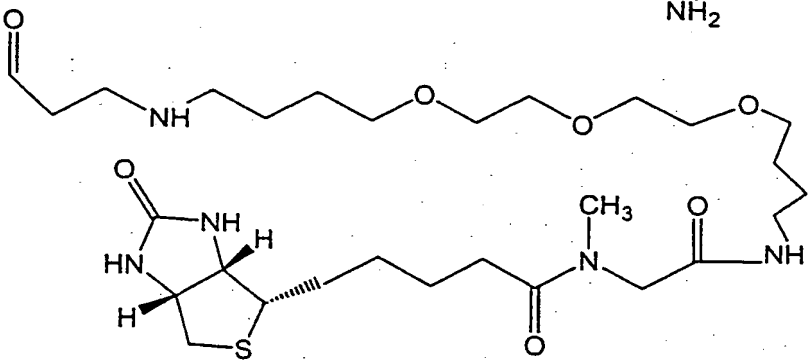
30, X =



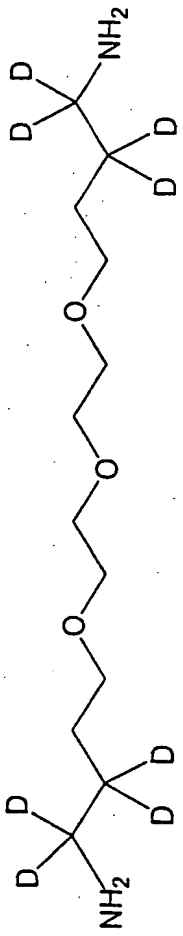
31, X =



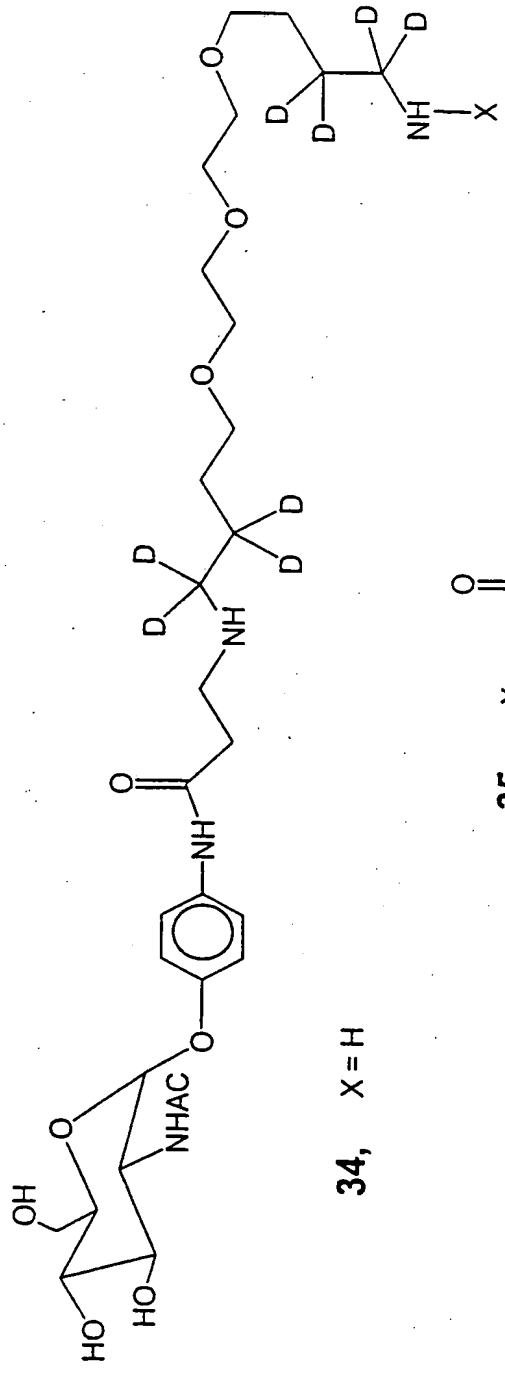
32, X =



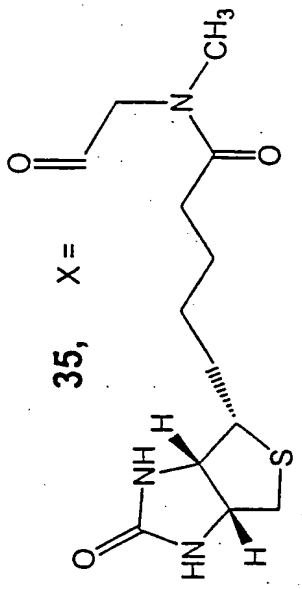
Scheme 21



33

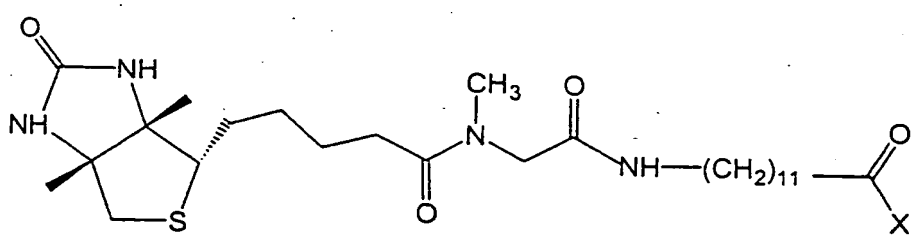


34, X = H

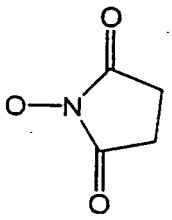


35, X =

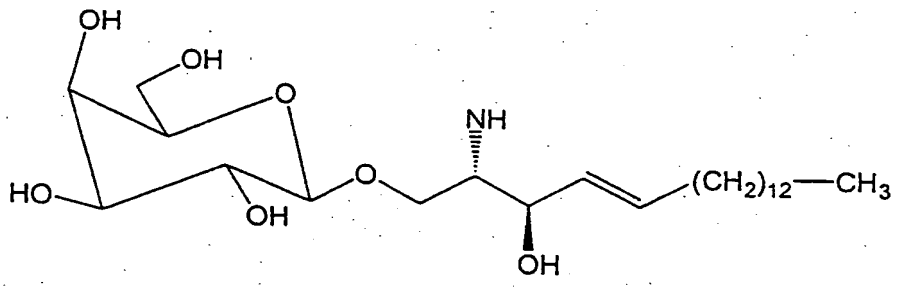
Scheme 22



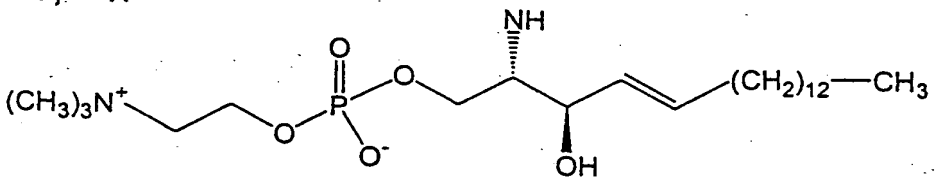
36, X = OH

37, X = 

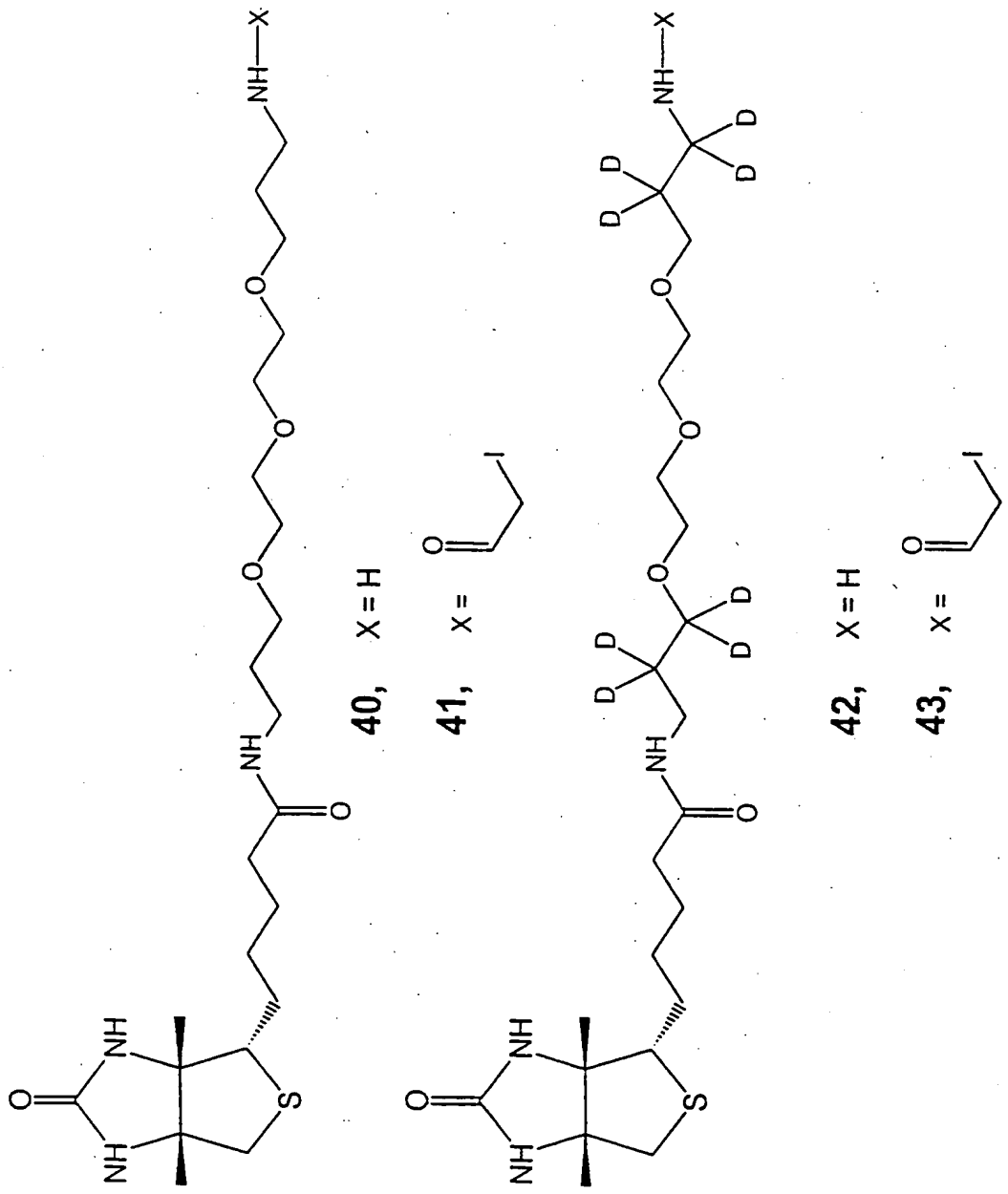
38, X =



39, X =



Scheme 23



40, X = H

41, X =

42, X = H

43, X =

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We claim:

1. A method for identifying one or more proteins or protein functions in one or more samples containing mixtures of proteins which comprises the steps of:

(a) providing an affinity tagged, substantially chemically identical and differentially isotopically labeled protein reactive reagent for each sample wherein the reagent has the formula:

A-L-PRG

where A is an affinity label that selectively binds to a capture reagent, L is a linker group in which one or more atoms can be differentially labelled with one or more stable isotopes and PRG is a protein reactive group that selectively reacts with certain protein functional groups or is a substrate for an enzyme;

(b) reacting each sample with one of the protein reactive reagents to provide affinity tagged proteins or affinity tagged enzyme products in the sample, affinity tagged proteins and enzyme products in different samples being thereby differentially labeled with stable isotopes;

(c) capturing affinity tagged components of the samples using the capture reagent that selectively binds A;

(d) releasing captured affinity tagged components from the capture reagent by disrupting the interaction between the affinity tagged components and the capture reagent; and

(e) detecting and identifying the released affinity tagged components by mass spectrometry.

2. The method of claim 1 wherein the affinity tagged proteins in the samples are enzymatically or chemically processed before or after their capture to convert them into affinity tagged peptides.

3. The method of claim 2 wherein the protein portion of one or more of the affinity tagged proteins are sequenced by tandem mass spectrometry to identify the affinity tagged protein from which the peptide originated.

4. The method of claim 1 wherein the protein portion of one or more of the affinity tagged proteins are sequenced by tandem mass spectrometry to identify the protein.

5. The method of claim 1 in which the amount of one or more proteins in the samples is also determined by mass spectrometry and which further comprises the step of introducing into a sample a known amount of one or more internal standards for each of the proteins to be quantitated.

6. The method of claim 1 wherein PRG is an enzyme substrate and the enzymatic

velocities of one or more enzymes in a sample are determined by quantitation of affinity tagged enzyme products and which further comprises the step of introducing into a sample a known amount of one or more internal standards for each of the affinity tagged enzyme products of an enzyme, the velocity of which, is to be quantitated.

7. The method of claim 1 wherein the released affinity tagged components are separated by chromatography prior to detecting and identifying the components by mass spectrometry.

8. The method of claim 1 in which a plurality of proteins or protein functions in one sample are detected and identified.

9. The method of claim 1 further comprising a step in which one or more of the proteins in a sample are chemically or enzymatically processed to expose a functional group that can react with an affinity tag.

10. The method of claim 9 wherein the PRG is an enzyme substrate for one or more enzymes, the deficiencies of which, are linked to a disease state.

11. The method of claim 9 wherein an affinity tagged, substantially chemically identical and differentially isotopically labeled enzyme substrate is provided for each enzyme that is to be detected and identified in a sample.

12. The method of claim 1 wherein PRG is a protein reactive group that selectively reacts with certain protein functional groups and a plurality of proteins are detected and identified in a single sample.

13. The method of claim 11 wherein two or more affinity tagged, substantially chemically identical and differentially isotopically labeled protein reactive reagents having different specificities for reaction with proteins are provided and reacted with each sample to be analyzed.

14. The method of claim 13 wherein all of the proteins in a sample are detected and identified.

15. The method of claim 1 wherein the relative amounts of one or more proteins in two or more different samples are determined and which further comprises the steps of combining the differentially labeled samples, capturing affinity tagged components from the combined samples and measuring the relative abundances of the affinity tagged differentially labeled proteins or the affinity tagged differentially labeled peptides originating from that protein.

16. The method of claim 15 which determines the relative amounts of membrane proteins in one or more different samples.

17. The method of claim 15 in which different samples contain proteins originating from different organelles or different subcellular fractions.

18. The method of claim 15 in which different samples represent proteins expressed in response to different environmental or nutritional conditions, different chemical or physical stimulii or at different times.

19. A method for determining relative expression levels of proteins in two or more samples containing proteins which comprises the steps of:

(a) providing an affinity tagged, substantially chemically identical and differentially isotopically labeled protein reactive reagent for each sample wherein the reagent has the formula:

A-L-PRG

where A is an affinity label that selectively binds to a capture reagent, L is a linker group which can be differentially labelled with stable isotopes and PRG is a protein reactive group that selectively reacts with certain protein functional groups:

(b) reacting each sample with one of the protein reactive reagents to provide affinity tagged proteins in the sample, affinity tagged proteins in different samples, being thereby differentially labeled with stable isotopes;

(c) combining the differentially labeled samples and treating the combined sample to cleave the proteins therein and to generate peptides;

(d) capturing affinity tagged differentially labeled peptides of the combined sample using the capture reagent that selectively binds A;

(e) releasing captured affinity tagged differentially labeled peptides from the capture reagent by disrupting the interaction between the affinity tagged peptides and the capture reagent; and

(e) detecting and identifying the released affinity tagged differentially labeled peptides by mass spectrometry; and

(f) measuring the relative abundances of the isotopically distinct ion peaks generated from an affinity tagged differential labeled peptide to determining relative expression levels of the protein from which the affinity tagged differential labeled peptide originated.

20. A reagent for mass spectrometric analysis of proteins which has the general formula:

A-L-PRG

where A is an affinity label that selectively binds to a capture reagent, L is a linker group which can be differentially labelled with stable isotopes and PRG is a protein reactive group that selectively that selectively reacts with certain protein functional groups.

21. The reagent of claim 20 wherein PRG is a sulfhydryl reactive group or an amine reactive group.

22. The reagent of claim 20 wherein PRG is an enzyme substrate.

23. The reagent of claim 20 wherein the A-L-PRG is soluble in a sample liquid to be analyzed.

24. The reagent of claim 20 wherein the linker is a cleavable linker.

25. The reagent of claim 20 which has the general formula:

A-B1-X1-(CH2)n-[X2-(CH2)m]x-X3-(CH2)p -X4-B2-PRG

where: A is an affinity label;

PRG is a protein reactive group; and

B1-X1-(CH2)n-[X2-(CH2)m]x-X3-(CH2)p -X4-B2 is a linker group wherein:

X1, X2, X3 and X4, independently of one another, and X2 independently of other X2 , can be selected from O, S, NH, NR, NRR'+, CO, COO, COS, S-S, SO, SO2, CO-NR', CS-NR', Si-O, aryl or diaryl groups or X1-X4 may be absent;
B1 and B2, independently of one another, are optional groups selected from COO, CO, CO-NR', CS-NR', (CH2)q-CONR', (CH2)q-CS-NR', or (CH2)q;

n, m, p, q and x are whole numbers that can take values from 0 to about 100, where the sum of n+xm+p+q is less than about 100;

R is an alkyl, alkenyl, alkynyl, alkoxy or an aryl group that is optionally substituted with one or more alkyl, alkenyl, alkynyl, or alkoxy groups; and

R' is a hydrogen, an alkyl, alkenyl, alkynyl, alkoxy or an aryl group that is optionally substituted with one or more alkyl, alkenyl, alkynyl, or alkoxy groups

wherein one or more of the CH2 groups in the linker can be optionally substituted with alkyl, alkenyl, alkoxy groups, an aryl group that is optionally substituted with one or more alkyl, alkenyl, alkynyl, or alkoxy groups, an acidic group, a basic group or a group carrying a permanent positive or negative charge; wherein one or more single bonds linking non-adjacent CH2 groups in the linker can be replaced with a double or a triple bond and wherein one or more of the atoms in the linker can be substituted with a stable isotope.

26. The reagent of claim 20 wherein the affinity label is biotin or a modified biotin.

27. The reagent of claim 20 wherein the affinity label is selected from the group consisting of a 1,2-diol, glutathione, maltose, a nitrilotriacetic acid group, or an oligohistidine.

28. The reagent of claim 20 wherein the affinity label is a hapten.

29. The reagent of claim 20 wherein PRG is a sulfhydryl-reactive group.

30. The reagent of claim 20 wherein PRG is an iodoacetylamide group, an epoxide, an α-haloacyl group, a nitriles, a sulfonated alkyl, an aryl thiols or a maleimide. .

31. The reagent of claim 20 wherein PRG is an amine reactive group, a group that reacts with a homoserine lactone or a group that reacts with carboxylic acid group.

32. The reagent of claim 20 wherein PRG is selected from the groups consisting of a amine reactive pentafluorophenyl ester group, an amine reactive N-hydroxy succinimide ester group, sulfonyl halide, isocyanate, isothiocyanante, active ester, tertafluorophenyl ester, an acid halide, and an acid anyhydride; a homoserine lactone reactive primary amine group, and an carboxylic acid reactive amine, alcohols or 2,3,5,6-tetrafluorophenyl trifluoroacetate.

33. The reagent of claim 20 wherein PRG is a substrate for an enzyme.

34. The reagent of claim 20 wherein PRG is a substrate for an enzyme the deficiency of which is associated with a birth defect.

35. The reagent of claim 20 wherein PRG is a substrate for an enzyme the deficiency of which is associated with a lysosomal storage disease.

37. The reagent of claim 20 wherein PRG is a substrate for β-galactosidase, acetyl-α-D-glucosaminidase, heparan sulfamidase, acetyl-CoA-α- D-glucosaminide N-acetyltransferase or N-acetylglucosamine-6-sulfatase.

38. The reagent of claim 20 wherein at least one of B1 or B2 is CO-NR' or CS-NR.

39. The reagent of claim 20 wherein X1 and X4 are selected from NH, NR, and NRR'+, X3 is O and all X2 groups are O.

40. The reagent of claim 20 wherein the linker contains a disulfide group.

41. The reagent of claim 20 wherein any atom of the linker may be substituted with a heavy isotope.

42. A reagent kit for the analysis of proteins by mass spectral analysis that comprises a reagent of claim 20.

43. The reagent kit of claim 42 that comprises one or more reagents of claim 20.

44. The reagent kit of claim 42 further comprising one or more proteolytic enzymes for use in digestion of affinity tagged proteins.

45. The reagent kit of claim 42 which comprises a set of substantially chemically identical differentially labelled affinity tagged reagents.

46. The reagent kit of claim 42 wherein the reagent is an affinity tagged enzyme substrate reagent.

47. The reagent kit of claim 46 which comprises a set of substantially chemically identical differentially labeled affinity tagged enzyme substrates.

48. The reagent kit of claim 47 further comprising a set of substantially chemically identical differentially labeled affinity tagged enzyme products.

Fig. 1A

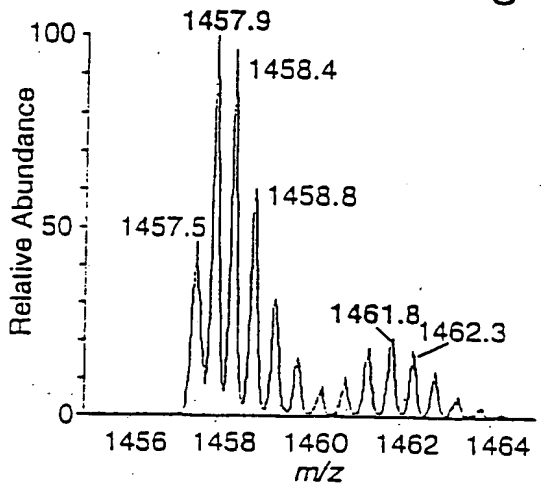
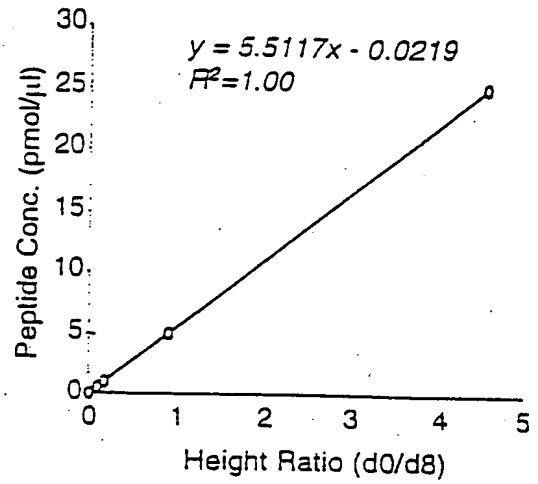
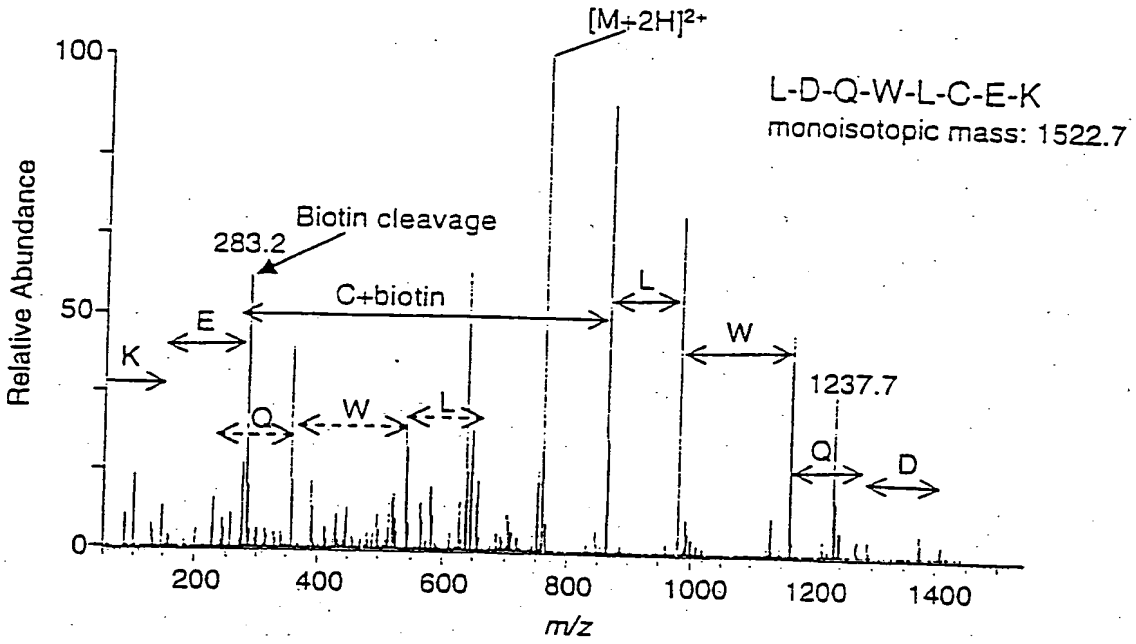


Fig. 1B



Standard curve generated with a cysteine-biotinylated peptide and quantitation by stable isotope dilution. A) Zoom-scan from an ion-trap mass spectrometer showing a 4 amu isotope distribution for the $[M+2H]^{2+}$ ions of the peptide modified with the isotopically light (1457.9 u) and heavy (1461.8) biotinylating reagents. The ratio (d0/d8) was 4.54. B) Curve generated from the analysis of isotope ratios from zoom-scans of 5 different concentrations of d0-labeled peptide measured in the presence of a known amount of peptide labeled with the isotopically heavy reagent.



Tandem mass spectrum of a cysteine-modified peptide from α-lactalbumin. Modification of the cysteine residue with the custom synthesized biotinylating reagent did not affect the ability of the Sequest computer program to correctly match this peptide to a database sequence.

Fig. 2

Fig. 3A

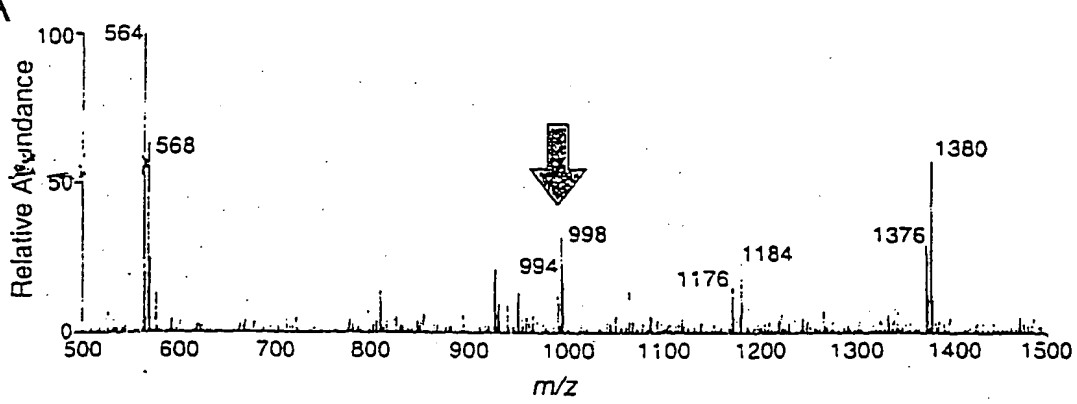


Fig. 3B

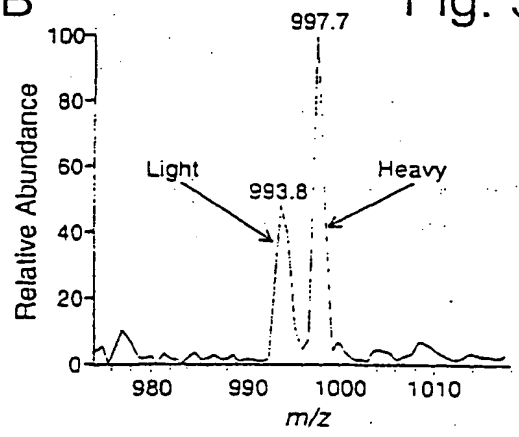


Fig. 3C

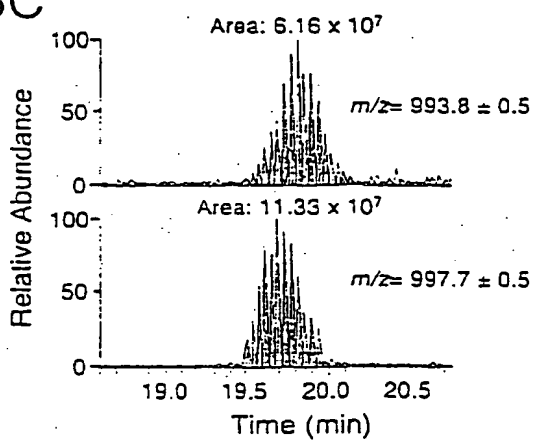


Fig. 4A

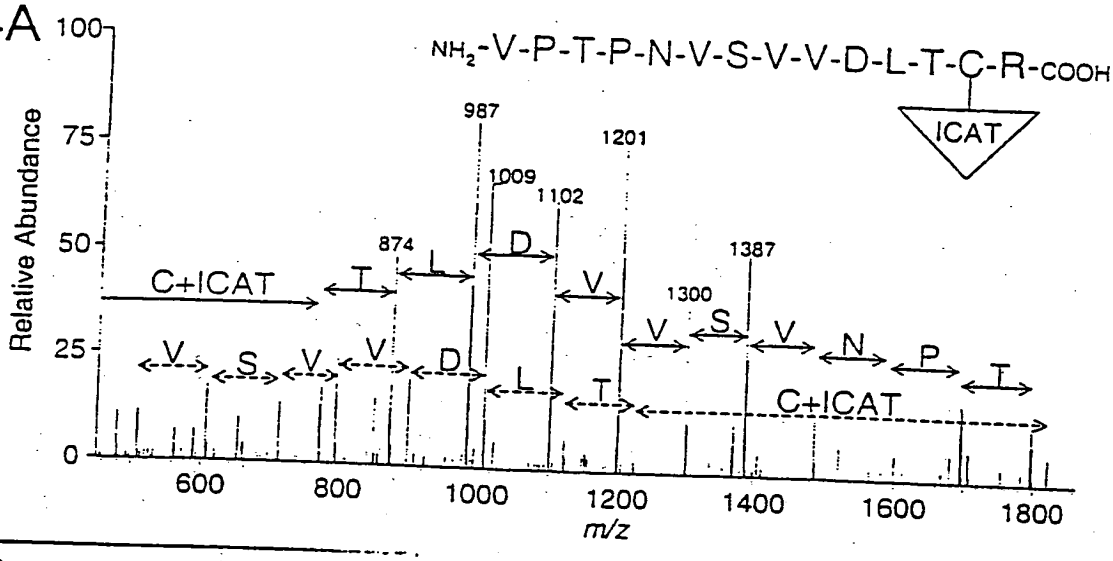
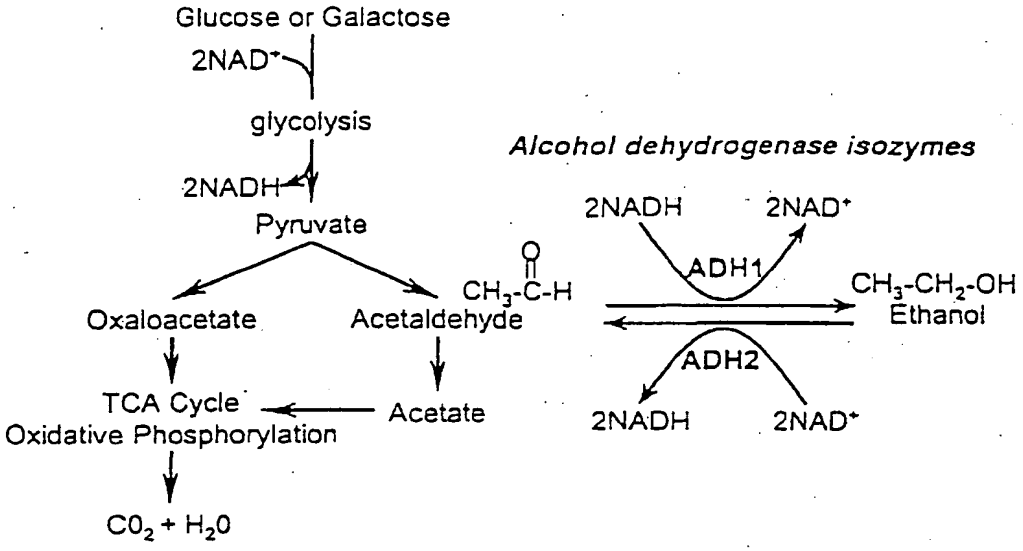


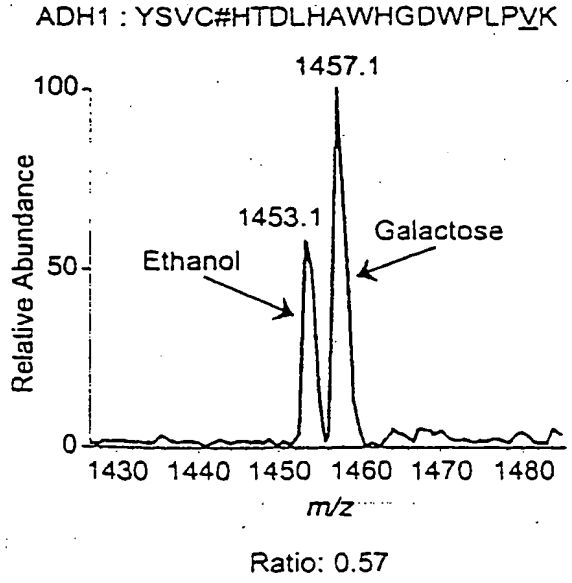
Fig. 4B

s0319_hlnavcid.0364.0364.2.out
amino acids = 93009033, # proteins = 290043, # matched peptides = 1973750
C:\LCQ\database\owl.v31.3, (C# +494.50)

#	Rank/Sp	(M+H)+	C*10^4	Ions	Reference	Peptide
1.	1 / 1	1994.3	4.4675	17/36	G3P_RABIT	(R)VPTPNVSVVDLTC#R
2.	2 /403	1995.1	2.7366	13/34	SLTRNGL	(E)LGKPVLTANQVTIWEGLR
3.	3 / 3	1995.0	2.5591	16/36	PLP_LACCA	(N)IANPNVYTETLTAATVCTI
4.	4 /209	1995.0	2.5335	14/36	A42912	(Y)LALLPSDAEGPHGQFVTDK
5.	5 /381	1995.1	2.4634	13/38	H69373	(L)ALLVLVAPAMAAGNGEDLRN

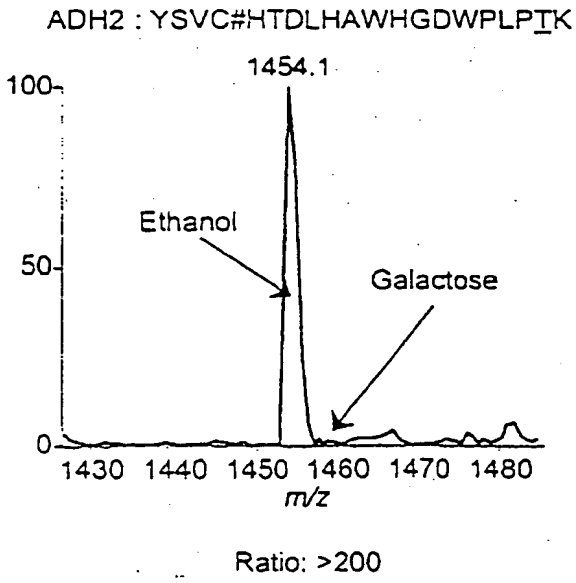
Fig. 5A





Ratio: 0.57

Fig. 5B



Ratio: >200

Fig. 5C

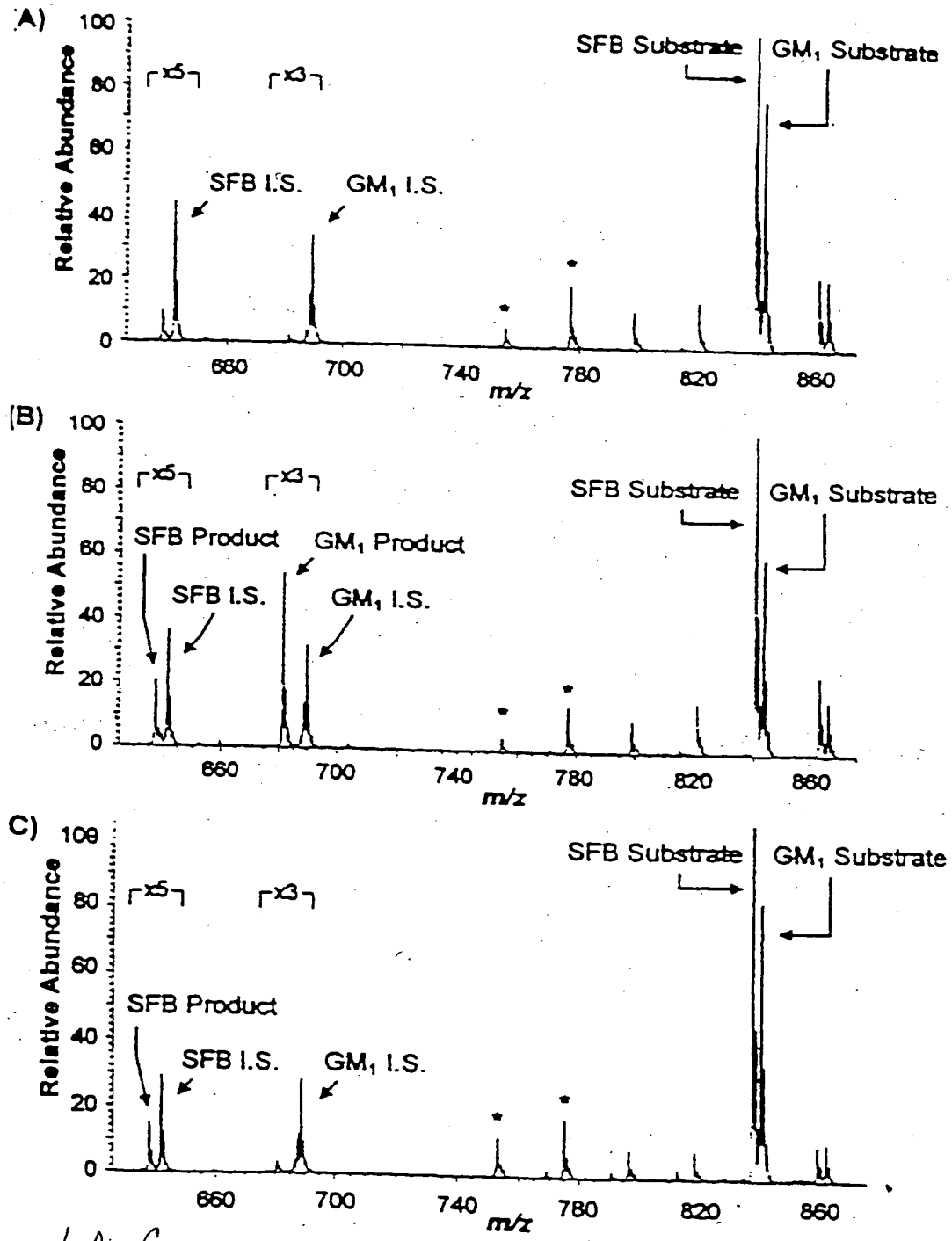

A)
Relative Abundance
SFB Substrate
GM1 Substrate
SFB I.S.
GM1 I.S.
m/z
(B)
SFB Product
GM1 Product
C)

Figure 6A-C

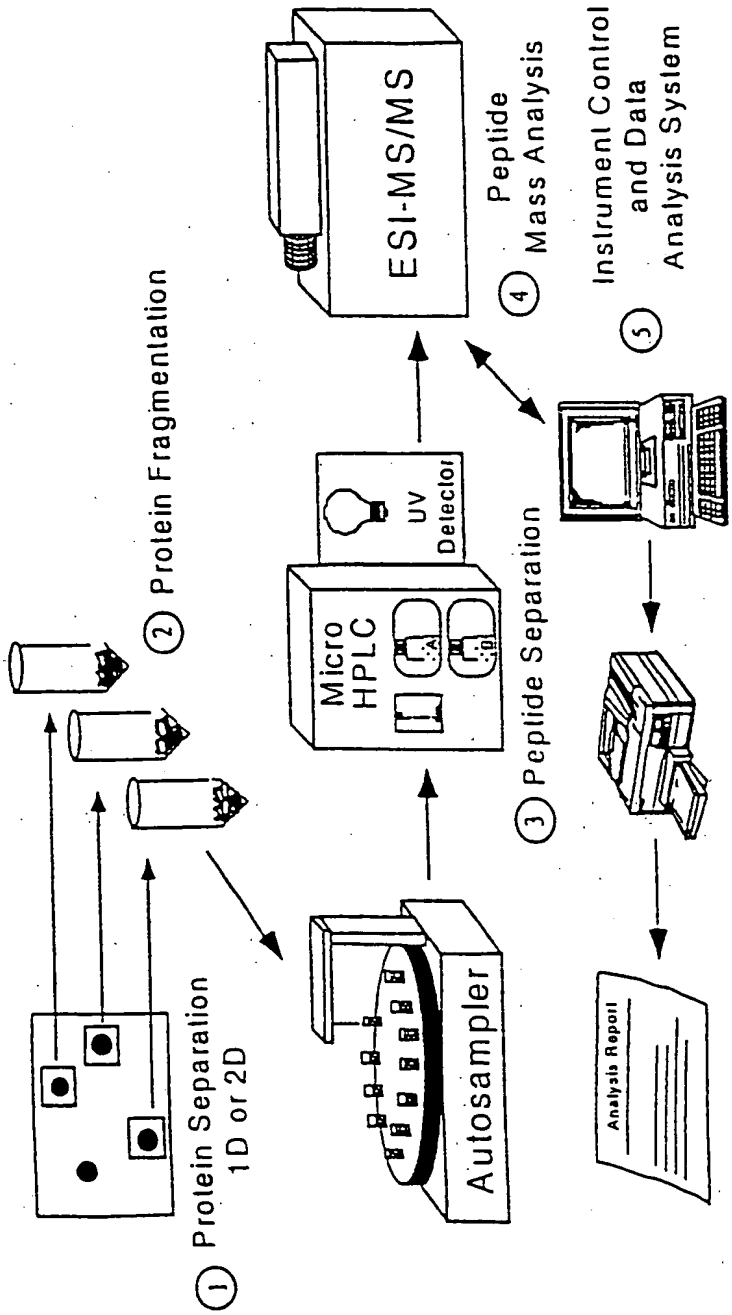


Figure 1 Schematic representation of the automated LC-MS/MS system. Proteins are typically separated by 1D or 2D SDS-PAG (1). Protein spots or bands are selected, excised and proteolytically cleaved with trypsin (2). Digests are loaded into an autosampler, which delivers them sequentially to the injection mechanism of a narrow-bore HPLC system (Michrom). The column gradient is automatically applied to separate individual peptides(3). Column eluate is sprayed directly into a mass spectrometer where sequence information from the peptides is collected (4). Recorded peptide masses and CID spectra are transferred to a data station for Sequest analysis, and a final summary of all identifications made for all samples originally loaded is sent to a printer (5).

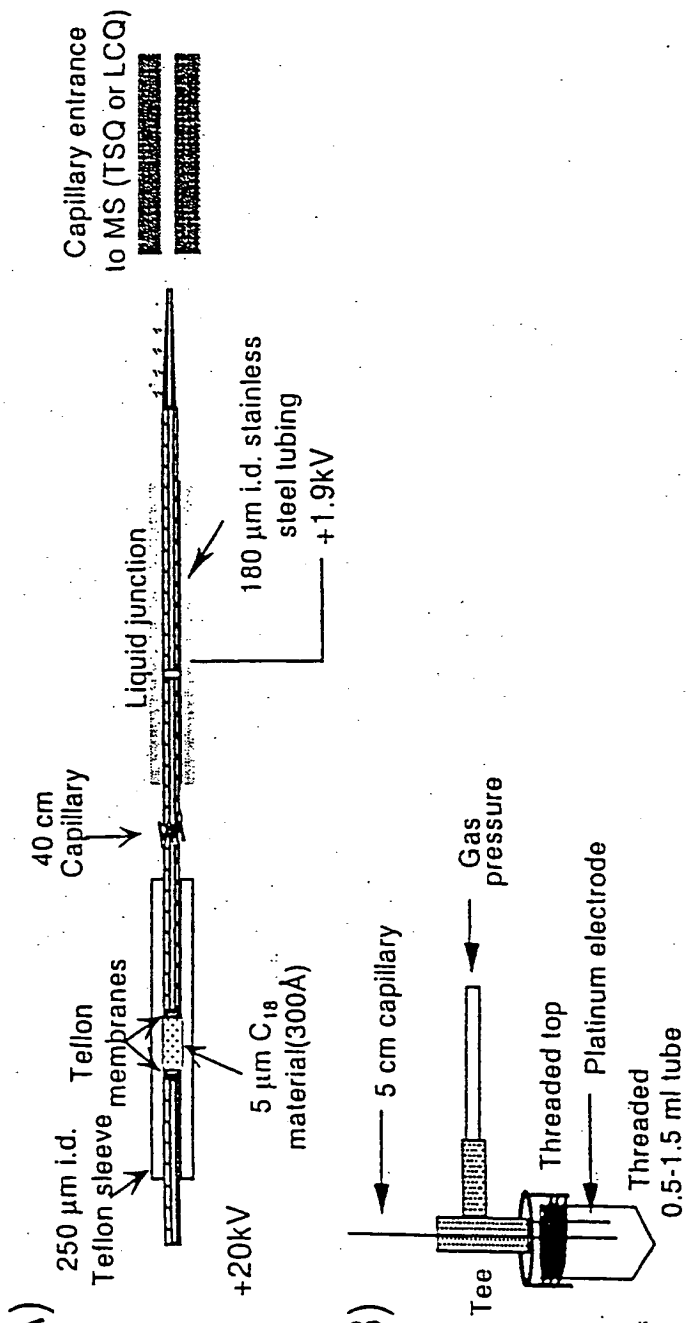


Figure 6 Schematic of the SPE-CE-MS/MS system. A) A fused silica capillary, typically of 50 µm i.d., is modified at the electrospray end with a liquid junction to establish electrical contact with the analytes inside the capillary. Approx. 5 cm from the end of the capillary, the SPE device is introduced. This consists of C_{18}-derivatized, large pore silica beads packed inside a 250 µm i.d. Teflon tubing with Teflon membranes at each end to hold the beads in place between the two fused silica capillaries. B) The injection end of the capillary is inserted into a sealed container which is maintained at a constant, slightly hyperbaric pressure in order to ensure constant flow. A platinum electrode is inserted through the cap, into the container, in order to allow the electrical contact to be made.